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(71) Applicant *(for all designated States except US)*: ALLIANCE PHARMACEUTICAL CORP. [US/US]; 3040 Science Park Road, San Diego, CA 92121 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : DEHAZYA, Philip [US/US]; 217 Galley Hill Road, Cuddebackville, NY 12729 (US).

(74) Agents: SIMPSON, Andrew, H. et al.; Knobbe, Martens, Olson and Bear, 620 Newport Center Drive, 16th Floor, Newport Beach, CA 92660 (US).

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(57) Abstract

A novel glycosylated form of albumin, designated EGA, is further characterized by its cell growth promoting activity. A process of isolation of human or bovine glycosylated albumin is described. The EGA fractions have been identified by serological histochemical and biological assays as well as lectin reactivity. The growth-promoting effect of EGA is directed to various transformed cell lines and primary cells of mammalian origin. Also described is a neutralizing antibody to the EGA of the present invention produced from a parental cell line. Novel compositions of EGA containing media are provided for cell, tissue or organ culture and diagnostic and therapeutic procedures are provided. Further, hybridoma cell lines producing inhibiting or neutralizing antibodies or antibodies or antibodies against EGA are described.

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A NOVEL SERUM GROWTH FACTOR

Field of the Invention

The present invention relates to an isolated and purified glycosylated form of albumin that is naturally occurring in blood and other bodily fluids and secreted from human liver cells in vitro culture. In accordance with the present invention there are described compositions and methods for the preparation and the use of the glycosylated albumin for cell growth, cell proliferation, enhancement of cell growth, cell differentiation and maturation, diagnosis and clinical applications.

Background of the Invention

In general, albumins are widely found in living material and are known as simple proteins defined as yielding only alpha-amino acids and their derivatives on complete hydrolysis. They are mainly characterized by solubility in water and dilute aqueous salt solutions.

As simple proteins, albumins are not conjugated. Conjugated proteins are defined as containing a protein molecule bound to an organic, nonprotein prosthetic group.

Among those albumins isolated into crystalline form, serum albumin has been determined to possess a molecular weight of 65,000 to 69,000 daltons. The pI values of serum albumin range from 4.7 to 4.9. The molecular weight of bovine albumin on the basis of its amino acid sequence has been estimated at about 67,300 daltons. (See also The Plasma Proteins, F.W. Putnam, ed., Vol. 1, 1975, Academic Press).

Serum albumin is the principal serum protein of mammalian organisms. In humans, the normal blood plasma range is 3.5-5.0 g of albumin/100 ml (or per dl). Albumin is also found in the extravascular spaces, the lymph, and in other biological fluids including amniotic fluid, bile, gastric juice, sweat and tears. Albumin is a major component of edema fluid. In fact, plasma albumin has been determined to have two major functions; one is maintenance of osmotic pressure; the other is transport f noncovalently bound substances. Such

substances comprise metals and other ions, bilirubin, amino acids, fatty acids, enzymes, hormones and drugs. More recently, albumin has also been characterized in diabetic conditions as capable of nonenzymatically binding several glucose molecules.

Albumin is produced substantially in the liver. In fact, liver cells or hepatocytes principally produce albumin. In a 70 kg human, the liver synthesizes about 12-14g albumin per day. Albumin has a half life of about 20 days in man. Albumin synthesis comprises the classical intracellular assembly of 575 amino acids. Its secretion is inhibited by ouabain. Albumin synthesis is stimulated *in vivo* by cortisone and thyroid hormones (thyroxine and triiodothyronine). Albumin synthesis *in vitro* appears dependent on osmotic pressure such that it is increased under hypotonic and decreased under hypertonic conditions.

Due to its small size, albumin leaks through blood vessel walls into extravascular space to be returned to the blood circulatory system by the lymphatic system. In patients with kidney disease, albumin is known to pass into urine, frequently leading to hypoalbuminia.

It has been commonly accepted that serum albumin (or plasma albumin) is alone among major plasma proteins in that it contains no carbohydrate (Eylar, J. Theor. Biol. 19, 89, 1965).

However, nonenzymatic glycosylation or glycation of albumin in patients suffering from diabetes mellitus has been observed as the consequence of the condensation reaction between relatively high concentrations of glucose and the free amino group at the N-terminus or epsilon-amino group of lysine residues exposed on the albumin molecule. The reaction proceeds by Schiff base formation between carbonyl groups of glucose and the free amino groups. However, the nonenzymatic formation of a ketoamino is slow and concentration dependent. Thus, prolonged hyperglycemia may increase the glycation level of albumin, in addition to that of many other serum proteins.

Moreover, the hitherto described glycated albumins appeared to contain nly one sugar residue per binding site.

Mor recently, Robert J. Peach, et al. (Biochimica et Biophysica Acta 1097 (1991) 49-54) characterized a new mutant form of albumin (Albumin Casebrook) carrying a single N-linked oligosaccharide which binds to Concanavalin A. The albumin variant constitutes about 35% of total serum albumin.

Summary of the Invention

Surprisingly, a minor glycosylated albumin fraction has now been discovered as generally present in mammalian blood. The isolated and purified protein exhibits a growth promoting and modulating effect in serum-free cultures of various cell types. While not wishing to be bound to any particular theory, the biological role of this new circulating factor appears connected with the growth and differentiation of a variety · f cells in mammals. Therefore, the factor has utility in the diagnosis and treatment of the processes of cell or tissue growth, development, differentiation and healing.

Acc rding to the present invention, there is provided an enzymatically glycosylated albumin (glycoalbumin EGA) that is isolated and/or purified from plasma or serum.

The growth factor in the form of glycosylated albumin according to the present invention provides a growth promoting or potentiating effect on serum-free cell cultures.

Further to the present invention, the instant glycoalbumin derived from biological fluids is characterized mainly as a single band moving on SDS/PAGE with an apparent molecular weight of at least 65,000 daltons. The isolated glycoprotein can be identified by serological means as an albumin, by specific glycosidase action or lectin reactivity as carrying oligosaccharides and consequently enzymatically glycosylated, and by addition to serum-free cell cultures as having growth stimulating, modulating or sustaining effect on various cell types.

Further to the identification of the purified fraction, the glycosylated albumin, EGA, was analyzed through sequencing

and computer assisted sequence matching. The N-terminal sequence of bovine EGA was found to be that of bovin albumin, namely, asp-thr-his-lys-ser-glu-ile-ala-his-arg, (see Brown, Fed. Proc. 34:591 (1975)), while the N-t rminal sequence of human albumin is asp-ala-his-lys-ser-gln-val-ala-his-arg, which is closely homologous to the bovine albumin sequence. F.W. Putnam, The Plasma Proteins, supra.

The present invention further provides a glycosylated albumin which binds to the surface of various cell types. In this context, the cell receptors for the glycosylated albumin of the present invention appears to be cell state-specific. The portion of the cell cycle exhibiting th most binding of albumin include the B_0/G_1 and early S phases.

The present invention is directed to a cell surface binding and growth activity which can be abolished by proteolysis or deglycosylation.

The glycosylated albumin fraction of the present invention also possesses Chinese hamster ovary ("CHO") cell growth promoting activity according to the present invention and is further characterized by isoelectric focusing (IEF) showing about four major bands and several minor ones of a pI of about 3.5-4.1.

The present invention is directed to an oligo-glycosylated bovine albumin characterized by an N-terminal amino acid sequence (ca. 10 amino acids) which is similar with the known bovine albumin sequence.

The instant enzymatically glycosylated albumin growth factor is characterized by the presence of at least one oligosaccharide chain, wherein the putative terminal sugar residue can be removed by neuraminidase followed by sequential removal of sugar residues with specific glycosidases.

Removal of N-linked oligosaccharide from the glycosylated albumin of the present invention abolishes the growth promoting activity.

The present invention is also directed to a process of purifying the oligo-glycosylated albumin (also designated as glycoalbumin or enzymatically glycosylated albumin, EGA) of

the present invention from blood or other body fluids that comprises the steps of removing cells, debris or coagulated material from said fluids, before further purifying the growth fact r by anion exchange chromatography, gel filtration, and pooling bioassay positive fractions and further purifying them by other means.

Another embodiment of the present invention is further directed to a process of purifying enzymatically glycosylated albumin (glycoalbumin) from blood comprising the steps of removing from blood plasma or serum proteins precipitated proteins in aqueous 50% saturated ammonium sulfate by centrifugation; dialyzing the supernatant containing unprecipitated matter against sodium acetate buffer, fractionating supernatant components by anion exchange chromatography using 3M NaCl salt step gradient elution in tris buffer at pH8.0; further fractionating the 3M NaCl eluate by size exclusion chromatography; collecting cell growth activating fractions in this buffer (pH8.6); and again fracti nating the active factor by linear gradient cation exchange chromatography while determining the activity by a bioassay.

The present invention further provides a growth factor comprising glycoalbumin as produced by hepatoma cell culture. In this context, the identification of the hepatoma-derived growth factor can be utilized in the growth regulation of the hepatoma cell culture. Moreover, the identification of the instant hepatoma-derived EGA growth factor can be applied to the diagnosis and treatment of hepatoma.

The present invention is also directed to the treatment of wounds using the glycoalbumin growth factor effect.

Another preferred embodiment of the present invention provides for a diagnostic test for liver pathology since liver is the main source for albumin and probably also EGA. The use of a combination of biochemical and immunochemical means as well as bioassay serves the diagnostic assessment of liver functi n.

The present invention is directed to a tissue and cell culture medium comprising a glycosylated albumin growth factor.

Moreover, in view of the growth-promoting activity of EGA, the present invention provides advantageous use of the EGA fractions as replacement of serum in compositions for cell, tissue or organ culture. Further in this context, the growth promoting effect of EGA is advantageously effective on precursor or differentiated primary cells or tissue.

In accordance with another aspect of the present invention there is provided a growth factor comprising enzymatically glycosylated albumin of the type occurring in the hepatoma cell line HEP G2 wherein the growth factor is substantially purified and is active in promoting the growth of mammalian cells *in vitro*.

In preferred embodiments, the growth factor of the present invention consists of a glycosylated albumin having an apparent M.W. of about 65,000 daltons on SDS/PAGE, serologically identified as albumin, binding to N-acetyl-D-glucosamine or sialic acid specific lectins, deglycosylated and/or desialylated by specific glycosidases, binding to a cell surface receptor, and activating and/or modulating cell growth.

In accordance with another aspect of the present invention, there is provided an enzymatically glycosylated albumin in isolated or purified form comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity. In a preferred embodiment, the glycosylated albumin is derived from bodily fluids, in particular, where the bodily fluids are mammalian, such as human bodily fluids. Alternatively, the glycosylated albumin may also be derived from cell, tissue or organ culture, *in vitro*. A particularly preferred source of the glycosylated albumin is from a culture of hepatoma cells.

The glycosylated albumin in a preferred embodiment possesses growth promoting and/or modulating activity, such as the albumin binding to cells. In addition, the binding is preferably related to a particular growth phase. The oligosaccharide moiety on the glycosylated albumin in a preferred embodiment comprises sialic acid or N-acetylglucosamine moieties. Moreover, the glycosylated albumin of the preferred embodiment possesses lectin-binding activity, such as binding activity with wheat germ lectin (T. vulgaris) or elderbark lectin (Sambucus nigra). In another preferred embodiment, the glycosylated albumin possesses an N-terminal amino acid sequence similar to or the same as human serum albumin. In still another preferred embodiment, the glycosylated albumin possesses serological similarity to albumin; i.e., is identified as albumin using standard serological tests.

In accordance with another aspect of the present invention, there is provided a composition for cell, tissue or organ culture media comprising the growth factor of the present invention. Preferably, the composition for cell, tissue or organ culture media is serum-free. Moreover, the composition in preferred embodiments additionally comprises additives selected from the group consisting of salts, buffers, and mineral sources.

In accordance with another aspect of the present inv ntion, there is provided an *in vitro* diagnostic test of liver function and/or pathology thereof comprising the steps of obtaining a sample of serum or plasma from an animal, is lating the protein fraction therefrom that has the properties of the glycosylated albumin of the present inv ntion, and quantitating the amount of the glycosylated albumin in comparison to a known quantity.

In accordance with another aspect of the present invention, there is provided a diagnostic test of liver function and/or pathology thereof comprising the use of the glycosylated albumin according to the present invention.

In accordance with another aspect of the present invention, there is provided a glycoprot in isolated from mammalian sources comprising an apparent molecular weight of about 65,000 daltons on SDS/PAGE, identified serologically and enzymatically as a glycosylated form of albumin having growth promoting activity. The glycoprotein is preferably a product of a hepatoma cell culture. For example, the product may be isolated from conditioned media of a hepatoma cell culture.

In accordance with another aspect of the present invention, there is provided a pharmaceutical composition comprising the glycosylated albumin of the present invention and a pharmaceutically acceptable carrier.

In accordance with another aspect of the present invention, there is provided a method of topical or percutaneous treatment of tissue that is in need of growth factor treatment in a warm blooded animal, which comprises administering to the animal an effective amount of the glycosylated albumin according to the present invention with a pharmaceutically acceptable carrier in a manner designed to deliver the glycosylated albumin to the tissue in need of treatment.

In accordance with another aspect of the present invention, there is provided a process for purifying the glycosylated albumin of the present invention which comprises salt precipitation, ion exchange chromatography, gel filtration, molecular sieve filtration or dialysis, lectin affinity chromatography, gel electrophoresis, and a bioassay for selecting fractions of the glycosylated albumin with growth promoting activity.

In accordance with another aspect of the present invention, there is provided a method for d termining whether a fraction of a fluid contains an enzymatically glycosylated albumin, the enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity, that comprises administering the fraction to a cell culture of Chinese hamster ovary cells with the

fraction, incubating the cell culture containing the fraction, and determining the presence or absence of the enzymatically glycosylat d albumin based on the growth of the cells, wherein an increase in the growth of the cells indicates the presence f the enzymatically glycosylated albumin and a lack of growth indicates the absence of the enzymatically glycosylated albumin.

In accordance with another aspect of the present invention, there is provided a neutralizing antibody against the glyc sylated albumin of the present invention. The neutralizing antibody may be against the growth factor derived from HEP-G2 or an enzymatically glycosylated albumin, the enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided a method of use of the neutralizing or inhibiting antibody in the immunoaffinity purificati n of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided a method of use of the neutralizing or inhibiting antibody in the treatment of a disease state wherein an enzymatically glycosylated albumin is overproduced, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In acc rdance with another aspect of the present invention there is provided an isolated antibody against the growth factor of the present invention.

In a c rdance with another aspect of the present invention there is provided an isolated antibody against an

enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided a method of use of the antibody in the immunoaffinity purification of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided a method of use of the antibody to specifically target cells involved in the production of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided an antibody to specifically target cells in a patient involved in the production of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity, comprising the steps of chemically linking the antibody to radionuclide to form a radiolabelled antibody; and administering the radiolabelled antibody to the patient, wherein the administration results in the delivery of the radiolabelled antibody to said target cells.

In accordance with another aspect of the present invention there is provided a method to purify an enzymatically glycosylated albumin from a liquid fraction, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity, comprising the steps of providing a column

containing a packing material linked to the antibody to create a column matrix; passing the liquid fraction through the column under conditions wherein the enzymatically glycosylated albumin preferentially binds to the antibodies on the column matrix; and eluting the enzymatically glycosylated albumin from the column.

In accordance with another aspect of the present invention there is provided a hybridoma cell line that secretes an inhibiting or a neutralizing antibody to an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and having mammalian cell growth promoting and/or modulating activity.

In accordance with another aspect of the present invention there is provided a hybridoma cell line that secretes an antibody against an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

Brief Description of the Figures

Figure 1 - Two-parameter analysis of FITC-EGA binding to CHO-K1 cell surface showing the majority of binding during the G_0/G_1S phases of cell cycle; a total of 19371 cells were examined;

Figure 2 - S-Sepharose chromatography elution profile of the 50% saturated ammonium sulfate supernatant from human plasma developed with a linear gradient of 0 to 0.1 M NaCl in 20mM sodium acetate buffer, pH 5.9, at 5°C as described below. The biologically active peak is indicated as EGA.

Figure 3 - An SDS-PAGE gel of purified EGA fractions containing 3, 1.5, 0.75, 0.375 µg protein per lane, respectively; Coomassie blue (C.B.) stain shows a single band;

Figure 4 - A Schiff-stained SDS/PAGE (10% w/v polyacrylamide) of partially purified bovine albumin column fraction (3M NaCl eluate from S-Sepharose) indicating

glycosylation; albumin bands are indicated by arrow;

Figure 5 - Western blot analysis of 150mM sorbitol eluate of human plasma fractionated by boronate affinity chromatography

A. Coomassie blue (C.B.) stained SDS/PAGE gel;
B. Western blot with anti-human antibodies;
C. Non-denaturing gel, C.B. stained;
D. Western blot with anti-human antibodies; albumin position indicated by arrows;

Figure 6 - Western blot analysis of glycosylated albumin which was purified from citrated human plasma fractionated by ammonium sulfate precipitation, S-Sepharose and gel filtration steps. Biologically active gel filtrate fractions were pooled, separated by non-reducing SDS/PAGE (with Mercaptoethanol) and transferred by the Western blot technique;

Lane 1: Anti-albumin antibody detection;
Lane 2: Application of biotinylated wheat germ l ctin followed by alkaline phosphatase reaction for detection;
Lane 3: Biotinylated S. nigra lectin and detection, as above.

Figure 7 - Human umbilical cord endothelial c lls in Matrigel medium, supplemented with EGA, display capillary-like processes; micrographs with (A) 200X and (B) 400X optical enlargement;

Figure 8 - Flow cytometric analysis of interaction of human buffy coat derived cells and FITC labelled EGA; and

Figure 9 - Isoelectric focusing gel (pH 3-10) stained with C.B. for analysis of the observed pI's of the biologically active fraction (=EGA) isolated from human plasma by ammonium sulfate precipitation and molecular sieving column chromatography (Sephacryl S-300 HR; Pharmacia). Arrows indicate the observed pH at the various points along the gel lane (left: IEF markers; right: biologically active S 300 HR pool) of the agarose IEF gel (FMC Bioproducts).

Detailed Description of the Invention

The glycosylated albumin of the present invention has been identified in, and isolated and purified from, mammalian blood or other biological fluids. The instant glycoprotein or EGA is reactive with certain lectins which are specific for sialic acid or glucose based sugar residues. Its glycosylation is further demonstrated by chemical staining procedures. The chromatographically purified EGA fractions exhibit a broad growth promoting activity in cultures of various cell lines, which activity has been found sensitive to proteases and glycosidases.

The instant glycoalbumin, EGA, crossreacts with anti-albumin antisera. EGA binds to various cells probably through cell surface receptors. The presence of these receptors in cells appears to be cell cycle dependent. In particular, fluorochrome labelled EGA binding has been observed on cells at the G_0, G_1 or early S phase of the cell cycle, suggesting a cell cycle dependent expression of receptors or receptor conformation (Fig. 1).

In this context, the present invention provides a bioassay for determining the activity of the instant EGA fraction whereby the growth promoting effect of EGA is observed in serum free CHO cultures. It is known that CHO (Chinese hamster ovary) cells will not adhere or grow on a solid matrix or tissue culture flasks in serum-free media. However, when serum-free media are supplemented with effective amounts of the instant glycosylated albumin, e.g., the human or bovine type (h-EGA or b-EGA), CHO cells will grow.

A further embodiment of the present invention provides an albumin from human or bovine biological fluids, preferably plasma or serum, which comprises glycosylated chains containing sialic acid or N-acetyl glucosamine residues, as well as galactosamine and galactosamine trimers (branched structures) and perhaps mannose.

The glycosylated albumin growth factor has been isolated from mammalian blood and bodily fluids. It can also be derived from the conditioned media of hepatoma cells indicating that the growth factor, like non-glycosylated

albumin, originates *in vivo* from the liver. EGA can be purified from its various sources including human or bovine blood serum or plasma and other bodily fluids.

Albumin comprises approximately 50% of the secr ted protein in conditioned media from HEP G2 (a human hepatocyte cell line, available commercially from the Wistar Institute, Philadelphia, PA, also commercially available from the American Type Culture Collection, Rockville, MD, as ATCC Deposit No. HB 80605). The EGA is an extremely minor component, comprising less than approximately 1% of all albumin in such media. It is believed that similar or smaller quantities of EGA are present in normal serum. Our methods of isolation and purification of the EGA of the present invention is described below.

Purification of Glycoalbumin

One preferred method for purifying the growth-promoting fraction of oligo-glycosylated albumin from bovine blood involves the following protocol. It will be appreciated that a variety of techniques that are well known to those of skill in the art can be used to separate proteins or peptides from biological fluids. Described below are techniques that are particularly effective in separating and/or purifying the EGA of the present invention. It will be understood that such techniques can be used alone or in combination depending on the bulk of starting materials and the desired purity desired in the products.

A. Salt Precipitation

Citrated blood is centrifuged to remove blood cells and platelets. The cell-free supernatant plasma is mixed with an equal volume of saturated ammonium sulfate or adjusted to 50% in order to remove a large portion of the prot in. The precipitated protein is centrifuged to a pellet. The supernatant liquid is decanted and dialyzed in 0.02M sodium acetate at pH5.9.

B. Column Chromatography

The dialyzed supernatant fraction is loaded on a S-

Sepharose (Pharmacia) anion exchange column (5x12 cm or 1.5 x 10 cm) and washed with 0.02M sodium acetate pH5.9. The active fraction is finally eluted with a linear gradient buffer of 0 to 0.2M NaCl at 5°C (Fig. 2). After dialysis, this fraction is loaded on a Q-Sepharose (Pharmacia) column, washed and developed with 0.02M tris buffer containing 1.0M NaCl. The active Q-fractions are pooled and further purified by gel filtration chromatography with Sephacryl S-300 Hr (Pharmacia), column bed size is approximately 1.5 x 70 cm at a flow of 1.5 to 2 ml/min at 5°C in 20mM Tris-HCl 150mM NaCl, pH7.5.

Another purification step is afforded by the use of boronate affinity chromatography, whereby fractions in 50mM Gly/NaOH (pH9) buffer are developed from column of P60 resin by step gradient with increased amounts of sorbitol in the glycine buffer.

The active EGA fractions are selected on the basis of the CHO-bioassay described below (Examples 12 through 15).

C. Gel Electrophoresis

The purified protein can be identified by non-denaturing, non-reduced polyacrylamide gel electrophoresis using the Laemmli's method but without first boiling the sample in SDS and/or mercaptoethanol. Further, the band corresponding to bovine albumin or glycosylated albumin fractions is further identified in typical SDS-PAGE gels which are 10% polyacrylamide in the separation gel portion (U.K. Laemmli, 1970, Nature, Vol.l 227, pp. 680-685). Moreover, Western blots of such slab gels have been prepared to probe the identity of EGA. (See Example 5 and Figs. 4-6).

Various methods for chromatographic purification of the glycoalbumin of the present invention are illustrated in the following Examples.

Example 1.

Purification of Glycosylated Albumin

Citrate-buffered human plasma was brought to 50% saturation with ammonium sulfate. The supernatant was fractionated on S-Sepharose (Pharmacia, Piscataway, NJ) (Bed size: 5 x 12 cm or 1.5 x 10 cm). Chromatography was

performed in 20 mM NaAc, pH5.9. Proteins were eluted with a linear gradient of 0 to 0.2 M NaCl at 5°C. Protein containing peak fractions were collected and pooled. Biologically active peak fractions were collected, pooled and loaded onto a Matrex-Gel P60 column equilibrated in 50mM glycine - NaOH, pH9.0. Non-specifically bound material was eluted with 1M NaCl. A step gradient of sorbitol in equilibration buffer was applied to develop the glycosylated albumin shown in the SDS/PAGE on lanes 1, 2, 3 and 4, containing 3, 1.5, 0.75, and 0.375 μg protein, respectively (Fig. 3). Western blots (see description in Example 5) of active EGA fractions isolated by S-Sepharose sorbitol gradient chromatography were prepared and tested for with anti-albumin antiserum and wheat germ as well as S. nigra lectins (Fig. 6, lanes 1, 2 and 3 respectively).

Alternatively or additionally, glycoalbumin can be purified by boronate affinity chromatography as shown in Example 2.

Example 2.

Boronate Affinity Chromatography

Chromatography is usually performed at 5°C. using a 1.6 x 9 cm. column packed with P60 resin (Amicon, Danvers, MA) and equilibrated with 50mM glycine/NaOH, pH 9.0. Sampl s to be fractionated were first dialyzed against a large excess of equilibration buffer and applied to the column at a flow of 1 ml/min. The column was developed using a step gradient of equilibration buffer containing varying concentrations of sorbitol. Prior to the application of the gradient, non-specifically adsorbed material was removed by washing the column with equilibration buffer containing 1M NaCl. A typical elution protocol are elution with 5, 10, 20, 50 and 100mM sorbitol, followed by elution with 20 mM NaAc/0.6M NaCl, pH 4.0. Pools of eluted material were dialyz d against 20mM Tris/150mM NaCl, pH 7.5 and tested for biologi al activity as described in Examples 12 through 15.

Example 3.

Alternative Purification of EGA

In anoth r preferred embodiment of the present invention, EGA was purified through salt precipitation with a 70% ammonium sulfate cut, to precipitate the protein as described above. Thereafter, the supernatant fraction was loaded on an S-Sepharose column in 125 ml of NaCl/20mM sodium acetate (pH 5.9) and eluted with 300mM NaCl. The active fraction, as determined in ur CHO-bioassay, was loaded on a HA Cartridge and eluted with 50mM sodium phosphate/TBS and the active fraction is again loaded on a Con A Sepharose column from which the active fraction eluted in 50mM α-methyl-mannoside/TBS. Thereafter, the above isolated active fraction from the Con A column was adjusted to pH 5.0 with polybuffer (1:80 diluted) and loaded on a Mono P Column and eluted with a pH gradient 5.0 to 2.5 in polybuffer (1:80)/2.5% Pharmalytes. From the Mono P Column, the growth active phase consistently eluted between pH 3.5 and 3.9.

Example 4.

Schiff Reaction of EGA

In order to identify the glycosylated albumin band(s) in gels (PAGE SDS), the gels are fixed in 10% acetic acid/50% methanol and subsequently rinsed in water for 1 hour. Thereafter, the gels were treated with 0.5% (w/v) periodic acid for two hours at room (ambient) temperature, then rinsed briefly with water. After rinsing, gels were immersed in 0.5% w/v sodium arsenite in 5% (v/v) acetic acid for 30 min., then in 0.1% w/v sodium arsenite in 5% (v/v) acetic acid for 10 min. Treated gels were immersed in Schiff's Reagent (Sigma) overnight in the dark, followed by rinsing in excess amounts of 0.6% (w/v) sodium bisulfite in 0.01M HCL (Fig. 4).

With ref rence to Fig. 9, slab gel isoelectric focusing of EGA reveal d several bands patterned in the range between pH 3.5 and 5.4, thereby indicating microheterogenesity within the isolated active fractions.

The glycosylated albumin fractions on polyacrylamide gels wer analyzed for identity and glycosylation by means of the Western blot technique.

Example 5.

Western Blot Analysis of EGA Preparations

Proteins were separated by polyacrylamide gel electrophoresis according to Laemmli (cited above) with or without SDS and blotted to nitrocellulose filter membranes (S&S Keene, N.H.) in an LKB Ultrablot apparatus in accordance with manufacturer's instructions. The immunoblots were blocked with 5% w/v non-fat dry milk before exposure to anti-albumin antibodies. Lectin-probed blots were blocked using 5% w/v fraction V bovine serum albumin (BSA). Antibodies and lectins were incubated with the membranes for 1-2 hours at appropriate dilutions. Bound antibodies were detected with secondary, anti-rabbit antibodies and chromophores as supplied in a kit (Amersham, Chicago, IL) (Fig. 5). The lectins obtained from Sigma Chemical Co. (St. Louis, MO) were biotinylated. After incubation and rinse the bound lectins were identified by WBT/BCIP chromatophores obtained in a kit Zymed (San Francisco, CA).

Western blots of EGA purified from human plasma through the S-Sepharose and gel filtration chromatography steps as described above, demonstrated reactivity with wheat germ lectin (Triticum vulgaris), which is specific for N-acetyl-D-glucosamine and tri-N-acetyl-D-glucosamine. Similarly, EGA reacted with sialic acid-specific lectin (Sambuca nigra) (Fig. 6). It was found that EGA also bound lectins derived from Caragana arborescens, specific for N-acetyl-D-galactosamine, and Lycopersicon esculentum, specific for tri-N-acetyl-D-galactosamine. The Western blot reaction with terminal mannose-specific lectin of Galanthus rivalis was barely detectable.

In order to determine the role of glycosylation in the growth promoting activity of purified glycosylated albumin, fractions were treated with endoglycosidases and exoglycosidases and then subjected to the bioassay. The

results further demonstrated the presence of oligosaccharide moieties on EGA.

Example 6.

Endoglycosidase Digestion

Referring to Table I, the Q-Sepharose purified albumin fractions from human plasma containing EGA was treated with endoglycosidases to remove the glycosylated moiety. Specifically, N-linked oligosaccharides were removed by incubation of the protein fractions with N-glycosidase F (Boehringer Mannheim, Indianapolis, IN) in 100mM tris-HCL, pH 7.0 for about 24 hours at 35°C. Protein concentration were approximately 1-4 mg/ml. The reaction mixtures contained 1 ml protein solution, 1.25U enzyme, and 5mM EDTA. Glycopeptidase F (Sigma) was used at a concentration of 2.5U with 1ml protein solution in 100mM sodium acetate, pH6.0 and incubated as above.

Neuraminidase treatment did not inhibit EGA activity, whereas digestions with endoglycosidase F plus N-glycosidase F were effective. Similarly, treatment of EGA with trypsin or chymotrypsin abolished growth promoting activity of EGA on CHO cells.

Removal of N-linked oligosaccharides was found to inhibit the growth promoting activity in CHO cells.

TABLE I

Enzyme Sensitivity of Q-9 Fractions (Bovine)

Treatment	Post-Treatment Growth of CHO Cells
Q-9 pH 5.1 control	+
Q-9 + neuraminidase	+
Cells + neuraminidase	+
Q-9 pH 7.0 control	+
Q-9 + endo F/glyco-F	-
Cells + endo F/glyco-F	+
Q-9 pH 8.0 control	+
Q-9 + trypsin	-
Q-9 + chymotrypsin	-
Cells + trypsin/chymotrypsin	+
EGA + O-glycanase	+/± Slightly diminished

Example 7.

Human EGA Activity: Testing for Sensitivity to O-Glycanase

Moreover, we also removed O-linked oligosaccharides by incubation of protein fractions with 12.5mU o-glycanase (Oxford Glycosystems, Rosedale, NY) per millimeter of protein solution in 5mM calcium chloride, thereby slightly diminishing bovine EGA activity. Neuraminidase (0.125U/ml protein solution) was also included to remove any terminal sialic acid residues blocking O-glycanase activity.

The degree of glycosylation and its effect on the cell growth promoting activity of glycosylated albumin was further explored by bioassay after digestion with exoglycosidases. The results are shown on Table I.

Example 8.

Exoglycosidase Sensitivity of CHO Cell Growth Promotion of Glycosylated Albumin of Bovine Plasma

Specifically, the exoglycosidase digestions of EGA fractions were assessed in 200 µl aliquots of fractions containing glycosylated albumin (1-2 mg/ml protein) incubated with the addition of the following enzymes: alpha glucosidase, 10U, 100mM NaAc, pH 6.8; beta glucosidase, 10U, 100mM NaAc, pH5.0; alpha galactosidase, 1U, 100mM NaAc, pH 6.5; beta galactosidase 10U, 100 mM Tris HCl pH 7.3; alpha mannosidase, 1U, 100mM NaAc, pH5.0; beta mannosidase, 0.25U, 100mM NaAc, pH5.0; alpha fucosidase, 0.25U, 100mM NaAc, pH5.0. All the exoglycosidase reaction mixtures also contained 5mM $CaCl_2$ and neuraminidase, 0.01U (Boehringer Mannheim). Controls contained buffer instead of enzymes and were treated identically. Incubations were held for 24 hours at 35°C.

The results (Table II) show that neuraminidase treatment alone did not inhibit the growth promoting activity of EGA as tested by bioassay of CHO cells, *in vitro*. Similarly, alpha galactosidase did not interfere with the EGA growth effect on CHO cells (*in vitro*). Results show that digestions in two

experiments with beta glucosidase, alpha mannosidase and beta mannosidase inactivated EGA activity.

TABLE II

Exoglycosidase Sensitivity of CHO Cell Growth Promotion of Glycosylated Albumin

ENZYME TREATMENT (a)	GROWTH OF CHO CELLS AFTER TREATMENT (b)
Alpha Glucosidase	+/- (c)
Beta Glucosidase	-
Alpha Mannosidase	-
Beta Mannosidase	-
Alpha Galactosidase	+
Beta Galactosidase	+/- (c)
Alpha Fucosidase	+/- (c)
Neuraminidase Alone	+

(a): all samples were treated with the listed glycosidase and neuraminidase concurrently, except as indicated

(b): two experiments

(c): +/-: variable effect or quantitative effect. Different results observed in two experiments.

It has been discovered that the growth promoting or activating effect of EGA is relatively broad as it is not necessarily limited to particular species. Moreover, a variety of cell cultures of different species origin have been tested and found to be receptive to growth stimulation by EGA (see Table III). Depending on the ambient conditions, developmental program or differentiation stage of the particular cells or cell lines, the instant EGA preparations may modulate growth by activating, retarding or inhibiting growth or even causing or aiding differentiation or quasi differentiation. The EGA used in the experiments depicted in Table III was derived from plasma, except for the hepatocyte cells, for which the EGA was derived from HEP G2. HEP G2 is a human hepatoma cell line ATCL, Deposit No. #B 8065. HEP G2 and EGA fraction derived therefrom as described in detail in connection with Example 1B.

TABLE III

Growth Promoting Activity of Human Glycosylated Albumin With Various Cell Types

CELL LINE	SPECIES	TISSUE	CELL TYPE	MEDIUM	SUPPLIER
CHO-K1	HAMSTER	OVARY	FIBROBLAST	F12/MCDB302	GIBCO
MDCK	DOG	KIDNEY	EPITHELIAL	MEM	GIBCO
MDBK	BOVINE	KIDNEY	EPITHELIAL	MEM	GIBCO
WI-38	HUMAN	LUNG	FIBROBLAST	DMEM	GIBCO
VERO	MONKEY	KIDNEY	EPITHELIAL	M199	GIBCO
*PRIMARY	HUMAN	UMBILICAL VEIN	ENDOTHELIAL	RSM210	VEC TEC
Clone 9	RAT	LIVER	HEPATOCYTE	DMEM	GIBCO

*with use of Matrigel as extracellular matrix.

**The DMEM was supplemented with 20% W/v HEP G2 conditioned media.

As shown in Table III, cell lines derived from various kinds of tissue that have been tested are stimulated to grow in the presence of the instant enzymatically glycosylated albumin (EGA). At concentrations as low as 2 μg/ml culture media cell adherence and replication has been observed. Therefore, cells thriving on EGA supplemented media include the epithelial, endothelial, and fibroblast varieties and even normal liver cells. The fact that normal liver cells thrive in the presence of the EGA of the present invention is particularly unexpected.

Example 9.

Cell Surface Localization of Glycoalbumin

Monolayers of Chinese hamster ovary (CHO-K1) cells were cultured for three days in basal media (Ham's F12; GIBCO) containing approximately 100 μg/ml bovine or human glycosylated albumin containing fractions isolated by S-Sepharose chromatography. After removing the media from the tissue culture flasks, the monolayers were exposed to 1mM disuccinimidyl suberate (Pierce, Rockford, Ill.) for 40 min.

at 0°C. Subsequently, the monolayers were rinsed four times in cold phosphate buffered saline. Cells were then incubated with anti-albumin antibodies directed against bovine or human albumin (USB, Cleveland, OH or Cappel, Durham, NC). Cell-bound antibodies were detected with an Amersham biotin - streptavidin secondary antibody detection kit using NBT/BCIP chromophores. Controls included cultures which were treated identically while omitting the antibodies to albumin or using preimmune IgG fractions.

Example 10.

Flow Cytometry

The apparent cell state specificity of EGA receptors was determined by a flow cytometric method. In one particular experiment, CHO cells were grown in the presence of S-Sepharose-purified glycosylated albumin fractions from bovine plasma wherein the albumin moiety was conjugated with FITC isoform 1 (Sigma), according to standard procedure. As described above, the cell monolayers were treated with disuccinimidyl suberate and released from the surface of culture flasks by means of enzyme-free disassociation buffer (GIBCO).

The isolated cells were fixed with 0.5% paraformaldehyde at 5°C, treated with 0.1% (v/v) Triton X-100 in PBS (phosphate buffered saline) for 5 min. at 5°C and washed with PBS. DNA was stained with propidium iodide (concentration of 50 μg/ml) after treatment of RNase (Sigma, 300U/mlPBS) for 30 min. at 37°C.

Cells were analyzed with a Coulter Epics flow cytometry instrument. In Figure 1, the X axis is proportionate to DNA fluorescence, the Y axis is proportionate to FITC-albumin (FITC-EGA) fluorescence. The arrow indicates the location of DNA fluorescence of cells in the $G_0/G_1/S$ portion of the cell cycle. Indeed, the majority of albumin fluorescent cells (Y-axis) is found on this part of the X-axis. In sum, the data support the conclusion that the receptors (binding EGA) are predominantly expressed at $G_0/G_1/S$ boundary (Fig. 1).

Taking reference now to Fig. 9 the following Example demonstrates the binding of EGA with human blood cells as measured by flow cytometry.

Example 11.

Interaction of Human Buffy Coat Cells With Glycosylated Albumin

Preparation of Glycosylated Albumin (EGA). EGA was purified by S-Sepharose and molecular sieve chromatography as described. The protein content was adjusted to approximat ly 2 mg/ml. The sample was dialyzed against an excess of 50mM glycine-NaOH, pH 9.0 buffer at 5°C, FITC (isomer 1-Sigma) was dissolved in DMSO at a concentration of 1 mg/ml. FITC was slowly and very gradually added in equal amounts to dialyzed EGA up to a concentration of 50μl FITC solution/ml EGA with constant agitation at 5°C. The solution was slowly stirred overnight at 5°C after addition of FITC was completed. Unbound fluorochrome was removed by molecular sieve chromatography (Sephacryl S300-HR-Pharmacia in 20mM Tris-150mM NaCl, pH 7.5 at 5C., TBS).

FITC-labeled EGA (protein: 13 mg/ml) was diluted 10 fold in Dulbecco's phosphate buffered saline. Buffy coat cells from human peripheral blood were prepared from EDTA anticoagulated blood by low speed centrifugation and washed several times with RPMI 1640 (GIBCO). The cell pellet containing several million cells was resuspended in 1ml of FITC-EGA and incubated 60 min. at 0°C. Freshly prepared DSS was added to cells as described for cell surface detection of albumin binding to CHO cells (as described above) and incubated at 0°C for 60 min. Cells were then washed with PBS, resuspended in 0.5% paraformaldehyde dissolved in saline and stored at 5°C, until analysis on an Epics Profile Analyzer (Coulter Electronics). As a control, buffy coat cells were treated with Tris-buffered saline instead of FITC-EGA.

In one experiment, 73,000 Control and 106,000 FITC-EGA treated cells were analyzed for fluorescence and for light scattering characteristics. The data obtained is illustrated

in Fig. 8. No significant fluorescence of erythrocytes was noted when the cells were subject to this procedure (data not shown).

The results shown in Fig. 8 clearly indicate a fluorescent cell population which is absent in the autofluorescence control. The FITC-EGA tagged cells were gated by light scattering and designated as a distinct population.

Example 12.

Glycated Human Albumin Effect

Moreover, no growth was observed in CHO cell cultures incubated in the presence of nonenzymatically glucose-substituted human albumin (glycated albumin, Sigma) at minimum concentrations of 200μg/ml using human albumin glycated with 1-4 hexose/mole of albumin.

The growth-supporting effect of EGA appears to be substantially similar to that of whole serum, e.g. fetal bovine serum, as demonstrated in the following experiments (Example 13). However, the results show that there may be some differences, too, particularly as observed through cell shape and/or cell appearance.

Example 13.

Morphological Effect on CHO Mutants

Further observations on the effect of glycosylated albumin were made on CHO mutants expressing altered amounts of cell surface proteoglycans. Cells lacking cell surface heparin sulfate or chondroitin sulfate exhibited similar morphological appearance in cultures whether grown in glycosylated albumin or FBS.

However, cells of a CHO mutant cell line 677, which over expresses chondroitin sulfate about 3 fold over the normal strain, exhibit an ovoid appearance in glycosylated albumin (EGA) containing media, in contrast to their mostly elongated fibroblast-like morphology in FBS supplemented media. Cells grown in glycosylated albumin fortified media were determined to bind the albumin as, e.g., by the immunocytochemical

procedure, as described above.

Example 14.

Human Umbilical Cord Endothelial Cells (h-UCE) (Fig. 7)

In addition to the above-recited observations on CHO cells, glycosylated albumin fractions were used to assess the EGA effect on human umbilical cord endothelial (h-UCE) cells when cultured on a differentiation system comprising RSM 210 (VecTec, Schenectady, NY) and Matrigel (Collaborative Biomedical Products, Medford, MA), a gelling growth matrix. Control incubations contained matrigel and medium 199,RSM 210 and 10% FBS. These human primary cell isolates were previously reported to form processes resembling capillaries (Madri, et al. J. Cell Biol. 106, 1988, 1375-1384.

While h-UCE cultures in the presence of matrigel with partially purified albumin (EGA) preparations showed capillary-like processes, cultures on Matrigel alone did not (see Fig. 3). EGA may therefore effect differentiation events as well as growth of primary, nontransformed cell lines.

Further, the growth effect studies appear to indicate that the glycoalbumin additive (EGA) promotes in serum-free primary cell cultures e.g. growth and differentiation of cultured monocytes to macrophages within days.

Example 15.

Human Monocyte-Macrophage Differentiation

Human peripheral blood was taken by venipuncture, citrated to prevent coagulation and centrifuged at low speed. The buffy coat was removed by aspiration and diluted twofold with phosphate buffered saline solution (PBS). It was then layered onto Ficoll-Paque (Pharmacia) at a ratio 2 vols: vol Ficoll-Paque. The mixture was centrifuged at 1200xg for 45 minutes at room temperature. The cellular contents concentrated in a discrete band at the Ficoll-Paque buffer interface. The cells were removed by aspiration and sedimented by low speed centrifugation, washed two times with RPMI 1640 (GIBCO) and diluted with this medium containing autologous human serum at 10% (v/v) concentration. The cells were then subjected to a simple selection regimen by placing

them into standard plastic tissue culture ware at an approximate density of $2x10^6$ cells/ml. After incubation of one hour at 37°C in 5% CO_2 in air, non-adherent cells and blood elements were removed by rinsing the plated cells with warm RPM1 1640 without serum two times. The remaining cells were incubated in medium containing autologous serum. After about 24 hours in culture, the cells were again rinsed with warm (37°C) serum-free medium and again incubated with serum supplemented medium.

In testing the various factions isolated from serum or plasma, the different quantities were substituted for serum in the suitable media, after several rinses of the cells with serum-free medium. Alternatively, the various fractions or purified factors can be tested by directly adding them to the cultures which have previously been rinsed with warm basal medium.

It has further been determined that, similar to albumin, the liver is also the major if not perhaps the only source for glycosylated albumin (EGA). This conclusion is supported by the discovery that glycosylated albumin is also expressed by hepatoma cells. In particular, HEP G2 cell cultures showed enriched presence of human glycosylated albumin, EGA, in conditioned media, as described below.

Example 16.

Albumin Expression by Hepatoma Cells

Conditioned media of cultured HEP G2 cells (human hepatoma - derived cell line) was fractionated by SDS-PAGE and western blots thereof subjected to incubation with antibodies to human albumin. The western blots were found albumin-positive. Based on standard comparison, there was an estimated concentration of 20-30 μg albumin/ml medium. Coomassie blue staining of the protein disclosed one major protein band migrating similarly to human albumin molecular weight standard (66,200 d.).

Other western blots of the hepatoma - conditioned media using lectin probes demonstrated reactivity of blot-identified

albumin bands suggesting the presence of sialic acid and N-acetylglucosamine in the albumin containing blots. Moreover, the glycosylation of the instant albumin is an intracellular event and not a consequence of peripheral circulation or, more particularly, by modifying serum enzymes.

To separate EGA from HEP G2 medium, the medium was subject ion exchange chromatography on a Q-sepharos column (as described for an S-sepharuse column in Example 1). The biologically active fractions were collected and pooled and on a hydroxylapatite column (HA cartridge) as discussed in Example 3. Thereafter, the biologically active fraction was loaded on sephacryl column for size exclusion chromatography.

The resulting biologically active fraction was analyzed on a non??? PAGE gel and also on ??? SDS-PAGE as discussed in connection with Example 1.

Example 17.

Assay of CHO Cells with Known Growth Factors

Interestingly, we have discovered that the single band appearing on SDS-PAGE is in fact four bands that are of close size. This is demonstrated through the use of a non-denaturing, discontinuous PAGE, where four bands based on their native charge and size are visible. How ver, when each of the four bands from non-denaturing PAGE are excised and again subjected to SDS-PAGE, the bands resolve to the same molecular weight range. The band was identified immunologically as albumin using commercially available monoclonal antibodies. The band was also r active with sialic-acid specific lectin (Sambuca Nigra), indicating the preserve of sialic acid residues.

In earlier studies, we found that the HEP G2 cell line under various conditions was capable of producing certain growth factors and other regulatory substances. To further establish that the EGA of the present invention is not one of these earlier identified growth factors or regulatory substances and to clarify the specificity of our CHO assay, we assayed CHO cells in microtiter wells in HEP G2 conditioned media in th presence of a variety of growth promoting or

regulating substances..

Generally, we inoculated 96 well microtiter plates with CHO cells (suspended in MCDB302 medium) having approximately 12,000 cells per well with HEP G2 conditioned medium (20 to 40 µl per well). Either growth factors (erythropoietin and epidermal growth factor) (available from Sigma or Upstate Biotechnology) or neutralizing antibodies (α-FGF, ß-FGF, transforming growth factor, and insulin-like 1 and 2 growth factor II) (available from Upstate Biotechnology) were diluted in PBS, following the instructions of the supplier to the concentrations indicated in Table IV, and added to the microtiter wells. The cultures were thereafter incubated and observed after 24 hours to assess whether attachment or initiation of growth were affected.

As will be seen from the results presented in Table IV, there was no observable effect on the CHO cell growth in the presence of either previously identified growth factors or neutralizing antibodies. The results also demonstrate that the growth factor of the present invention is different than the growth regulatory substances that have previously been described as secreted by the HEP G2 cell line. Although HEP G2 cells are not known to express hepatocyte growth factor, CHO cells that are receptor minus for this protein do not respond to it.

TABLE IV

RESPONSE OF CHINESE HAMSTER OVARY (CHO) CELLS TO GROWTH EFFECTS OF HEP G2 CONDITIONED MEDIA AND OTHER FACTORS

AGENT	CONCENTRATION	EFFECT ON CHO CELLS
Erythropoietin	0-50 mU/ml	no growth
Epidermal Growth Factor	0-10 ng/ml	no growth
Insulin-like Growth Factor 1 & 2	6.25:100ng/ml	no effect[1]
α-FGF (neutralizing antibody)	10 ug/ml antibody concentration	no effect[1]
ß-FGF (neutralizing antibody)	10 ug/ml antibody concentration	no effect[1]
Transforming Growth Factor (neutralizing antibody)	10 ug/ml antibody concentration	no effect[1]
Insulin-like Growth Factor (neutralizing antibody)	10 ug/ml antibody concentration	no effect[1]

1 Presence of neutralizing antibody did not inhibit CHO cell growth by HEP G2 conditioned media.

Example 18.

Production of Hybridomas and Antibodies to Inhibit EGA

We prepared antibodies to parental hybridoma cell lines of HEP G2 in order to determine if such antibodies could inhibit or neutralize the growth of CHO cells in HEP G2 conditioned media.

Hybridomas were prepared according to conventional techniques. See, for example, Cohen et al., J. Immunological Methods, 117:121-129 (1989). Female A/J mice were immunized with 100 µg of a bovine albumin antigen consisting of materials from a column fraction containing semi-purified EGA dissolved in PBS and mixed with Freud's complete adjuvant. The mixture (1:1) was injected interperitoneally. Seven days later, the mice were boosted with antigen mixed with incomplete adjuvant (1:1), with heat denatured antigen alone

1 week later, and then on three sequential days during the fourth week, after which the mice were killed and the spleens removed.

The spleen cells were fused with a mouse myeloma line, Sp2/0-Ag14, (ATCC CRL 1581; ATCC CRL 8287), and hybridoma colonies were established according to conventional techniques. E.g., Kennet et al., "Monoclonal Antibodies: Hybridomas: A New Dimension in Biological Analyses", Plenum Press, New York (1982). The resulting hybridoma colonies with bovine albumin binding activity and EGA binding activity were cloned at least four times by limiting dilution.

Col nies were screened for bovine albumin binding activity by ELISA, using 100 µl of hybridoma supernatant, using conventional techniques. E.g., Cohen et al., J. Immunological Methods, 117:121-129 (1989). From this screening, twelve parental cell lines were selected, with each parental cell line producing antibodies to the antigen complex. Each parental line was grown to a density of approximat ly 10^6 cells/ml in DMEM with 10% fetal calf serum (FCS) at 37° C with 5% CO_2 in air in 75 cm^2 flasks with 10 ml of growth media in each. Medium was harvested after a week of growth and passed over a column (volume 2 ml) containing agarose coupled antibodies to murine IgG and IgM. The column was washed with 0.15 M NaCl, then with 2 ml 0.05 M glycine-HCl, pH 2.5, to elute the uncoupled antibodies from the column. The eluent, containing the antibodies, was neutraliz d and the solution was dialyzed against cold TRIS buffered saline (TBS; 0.02 M TRIS/0.15 M NaCl, pH 7.5).

HEP G2 conditioned media was serially diluted in DMEM in 96 well microtiter plates. Aliquots (40 µl) of the dialyzed antibodies were added to each well containing the HEP G2 conditioned media. The plates were incubated for 1 hour, after which a suitable number of CHO cells were added to the wells (approximately 12,000 cells/well). Thereafter, the plates were incubated for approximately 24 hours at 37° C in 5% CO_2 in air and scored for CHO cell attachment and growth.

The results are depicted in the following Table V.

TABLE V

ACTIVITY OF ANTIBODIES SECRETED BY PARENTAL CELL LINES AGAINST BOVINE ALBUMIN ANTIGEN IN HEP G2 CONDITIONED MEDIUM

			CONDITIONED MEDIA					
Antibody	ELISA	Dot Blot	Undiluted	1:2	1:4	1:8	1:16	1:32
1G6	+	+	++	++	+	+	+	+/-
12.1007	+	-	Not Done					
12.3H4	+	-	Not Done					
12.9E12	+	+	++	++	++	+	+	+/-
12.6G47	+	+	+/-	+/-	+/-	+/-	-	+/-
12.1F11	+	+	+	+	+	+	+/-	+/-
12.8H10	+	+				+	+/-	+/-
12.9G6	+	+				+	+	+/-
12.1088	+	+				+	+/-	+/-
12.3C8	+	+				+	+/-	+/-
12.9D5	+	+				+	+/-	+/-
12.9C5	+	+				+	+/-	+/-

The Dot Blots and enzyme-linked immunosorbent assay (ELISA) were conducted according to standard techniques as, for example, described in "Antibodies - A Laboratory Manual" Harlow and Lane, Eds., Cold Spring Harbor Press (1988).

It will be appreciated that the antibody, 12.6G47, exhibited certain inhibitory or neutralizing activity on the growth of the CHO cells in the HEP G2 conditioned medium. Neutralizing antibodies of this type can be produced, either from the parental cell line producing the antibody 12.6G47, or monoclonals can be prepared through conventional techniques of priming mice with pristane and interperitoneally injecting mice with these or other neutralizing antibody hybrid cells to enable harvesting of the monoclonal antibodies from ascites fluid.

Example 19.

Preparation of Antibodies Against EGA

More specific antibodies can also be prepared in a manner

similar to the methods described in Example 18. For example, through immunizing the mice with the EGA, instead of the bovine albumin complex, it is expected that spleen cells harvested from such mice and fused with a myeloma cell to form a hybridoma line or lines will secrete antibodies to the instant EGA. Such antibodies will facilitate selective recognition between the EGA of the present invention and non-glycosylated, and possibly non-enzymatically glycosylated, albumins in sera or other bodily fluids.

Monoclonals antibodies can then be prepared through conventional techniques of priming mice with pristane and interperitoneally injecting mice with the hybrid cells to enable harvesting of the monoclonal antibodies from ascites fluid.

This was recently demonstrated in a related context by Cohen et al., who developed an antibody (A717) specific for non-ezymatically glycosylated albumin. Such antibody was capable of recognizing and facilitating the separation of non-enzymatically glycosylated albumin from serum albumin. See Cohen et al., J. Immunological Methods, 117:121-129 (1989).

Antibodies or monoclonal antibodies are expected to be useful in the efficient purification of the EGA of the present invention, as well as *in vivo* or *in vitro* tests relating to cell growth, regulation, and differentiation, in particular, those f liver cells. Moreover, such antibodies allow the tracking f liver function through the ability to specifically target and label the portions of the liver undergoing production of the EGA of the present invention.

Further, the potential therapeutic ramifications of antibodies against the EGA of the present invention are many. In situations of over-production of the glyco-albumin of the present invention, for example, as probably occurs in certain liver carcinomas, inhibiting or neutralizing antibodies her inabove described can be utilized to target such over producing cells and effectively inhibit hepatocyte over production. Additionally, through linking such antibodies to

effective chemotherapeutic agents or radionuclides, the antibodies can be used as direct anti-cancer agents. In each of the above instances, the techniques discussed are commonly used by those of skill in the art.

It will be appreciated that antibodies are regularly used in immunoaffinity techniques to purify peptide and protein from complexes based on their binding affinities. Accordingly, in one aspect of the present invention, the neutralizing or inhibiting antibodies of the present invention are used to purify the EGA of the present invention. This purification is accomplished through binding the antibodies to an appropriate support, for example, CNBr-activated sepharose. A column can be readily prepared for purification of the EGA of the present invention. The activity of fractions eluted from the column can be readily determined in our CHO cell-bioassay. Such techniques will allow the use of the neutralizing antibody in the immunoaffinity purification of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

The purification of hepatoma culture - derived glycosylated albumin fractions from a cell line such as HEP G2 avails itself to production of human glycosylated albumin growth factor through recombinant DNA technology. For instance, gene amplification can provide substantially increased *in vitro* production of EGA.

Example 20.

Growth Compositions Containing EGA

A preferred embodiment of the present invention relates to compositions containing glycosylated albumin (EGA) that enhance growth and productivity of cells and tissues in culture media at concentrations of EGA varying from 1-300 µg/ml medium. As will be appreciated, such concentrations are enhanced relative to the normal concentrations of EGA naturally occurring in the compositions from which the EGA is

derived. Media may be prepared with enhanced concentrations of EGA from 2 to 1000 times that which naturally occurs, and preferably at least 2 to 5 times, more preferably, 5 to 10 times. However, concentrations 10 to 100 and even 100 to 1000 are expected to be highly effective. Furthermore, in preferred embodiments, the EGA of the present invention is prepared so as to be substantially separated from normal or non-glycosylated albumin. In this way, whereas the general ratio of EGA to albumin is less than 1% to 50%, the compositions of the present invention contain significantly enhanced levels of EGA to albumin.

As will be appreciated, cell growth medias may be prepared using conventional techniques and reagents. However, rather than supplementing the media with serum or fetal calf serum, as is normally done, the growth media compositions of the present invention may be supplemented with EGA. Thus, the growth compositions prepared in accordance with the present invention generally include conventional reagents, such as, salts, buffers, amino acids, sources of vitamins, minerals, essential metals, and carbohydrate, supplemented with EGA.

Example 21.

Wound Treatment

Similarly, compositions of EGA may have efficacious use in the treatment of wounds in connection with its demonstrated growth promoting effect of cells. Particularly in view of the known presence of albumin in edemas or wounds, it is believed that the EGA of the present invention may play a biological role in wound repair. In wound repair, a medicament or pharmaceutical preparation containing an enhanced level of the EGA of the present invention in a pharmaceutically acceptable carri r will be highly efficacious. Typically, the compositions comprise an isolated and purified or recombinantly manufactured EGA fraction from a suitably homologous species and a pharmaceutically acceptable carrier for topical use.

The dose of the EGA fraction used in the treatment of wounds, especially on the skin, will vary with the relative

efficacy of the EGA material. However, as a general guide, applicable concentrations may range from about 1 μg/ml to about 500 μg/ml. As discussed above, such amounts represent an enhanced concentration of EGA relative to the source from which the EGA was derived. The compositions may be administered more than once a day, for example, sev ral times a day, continuing over several days or weeks as long as such treatment is needed.

Within the preferred range indicated above, the EGA preparation is not expected to have toxic effects. The preferred embodiment of the topical treatment of wounds is intended as utilizing EGA fraction derived from human serum or plasma for treatment of human skin. Similarly, in the case of other warmblooded animals, the EGA fractions of the invention are isolated from the identical species which is to be treated in order to avoid possible rejection or other immuno-toxic effects.

For topical and percutaneous administration, the preparations may also be presented as an ointment, cream, lotion, gel, spray, aerosol, wash, skin paint or patch.

A composition of an effective amount of EGA fractions for topical or percutaneous use may also contain antioxidants, vitamins, amino acids, carbohydrates, lipids, trace metals, minerals, physiologically amenable salt solutions or buffers, as well as antibiotics and tissue activity modifying agents as tissue plasminogen activators other factors.

Example 22.

Liver Function Diagnostic Test

Because of the apparent close connecti n between EGA production and liver cells (i.e., EGA is a demonstrated mitogen for liver cells and is produced by hepatocytes), the EGA of the present invention provides a uniqu diagnostic test of liver function and pathology thereof. The diagnostic tests in accordance with the present invention preferably include an assay of circulating glycosylated albumin (EGA) fractions and ratio or percentage of EGA to total or unglycosylated albumin

or pathological change thereof. The diagnosis may depend on procedures utilizing serological or chromatographic measurements including isolation, purification, and biological activity assays. The lectin adsorbancy test described above also serves to separate EGA from other, nonenzymatically "glycated" albumins. In the alternative, liver biopsies may prove amenable to screening for EGA production in culture.

Example 23.

Liver Carcinoma Diagnostic Test

It is expected that liver cell carcinomas may over-express EGA. Indeed, this has been demonstrated with HEP G2. Thus, another useful embodiment of the present invention is a diagnostic test for such liver carcinomas. In connection with such diagnostic test, a assay is run to determine the levels of EGA being produced by liver cells. Typically, the assay includes a combination of serological and chemical methods, as in, for example, immunoassay and lectin binding of circulating EGA. In addition, the assay might include liver biopsy and cell culture techniques.

In another embodiment, the assay comprises the use of an antibody or monoclonal antibody directed against EGA, as prepared above in connection with Examples 18 and 19. In a preferred embodiment, bodily fluids or blood serum of a patient is isolated and prepared for an immunoassay with an antibody directed against EGA. In preparing the serum, generally it will be dialyzed and subjected to chromatographic purification techniques of the type previously described for purification of EGA, in order to minimize cross-reactivity and enhance the quantity of EGA present. Preferred preparation steps are described in connection with Examples 1-5.

Thereafter, the EGA reactive fractions can be assayed and the levels of EGA quantitated through such techniques as ELISA, Western blots, SDS PAGE, or non-denaturing PAGE, to name a few. It will be appreciated that similar tests have recently been developed for assaying the levels of α-fetoprotein, which is expressed and secreted by certain liver

carcinomas.

As will be understood, such the antibodies of the present invention can also be readily radiolabelled through a variety of chemical linking reactions, such as biotinylation, cross-linking reactions with glutaraldehyde, or direct radiolabelling, for example. Appropriate radionuclides include I-125, I-131, among others. Once the radiolabelled antibody is formed, it can be used to specifically target cells that are over-producing EGA.

In a preferred embodiment, the radiolabelled antibody is administered to a patient and his or her body may be scanned for the locations of high concentrations of radiolabel.

Example 24.

Treatment of Liver Carcinoma with EGA

It has been recently demonstrated that certain liver carcinomas express and secrete α-fetoprotein at enhanced levels. It was found that liver cells in mice having liver cell carcinoma of these sorts could be transplanted to nude mice, such that the nude mice would become infected with the carcinoma. The nude mice, then, possessed the carcinoma that expressed and secreted α-fetoprotein. The nude mice were administered antibodies directed against α-fetoprotein and their tumors were inhibited. If antibody treatment was continued, the mice could be forced into remission.

Thus, it is believed that other autocrine driven liver carcinomas may be similarly controlled.

Accordingly, in a preferred embodiment, the invention is used to treat those liver carcinomas that express and secrete EGA as an autocrine driven disease state. The treatment comprises administering antibodies prepared in connection with Examples 18 and 19 that are directed against EGA. In this manner, autocrine carcinomas that are driven by EGA can be controlled.

Example 25.

Liver Cell Reconstitution with EGA and Solid Phase Implantable Supporting Structure

In another embodiment of the invention, *in vitro* liver cell reconstitution can be accomplished. In this embodiment, a cell culture of liver cells is prepared in a non-serum containing medium supplemented with EGA. The liver cells will grow, divide, and differentiate, as described in connection with Example 8 and Table III.

Moreover, we have developed a procedure for the preparation of an implantable device loaded with viable liver culture. A solid phase porous support structure is provided. The support structure is preferably a porous Teflon® (a brand name for polytetrafluoroethylene) or polyethylene material, although other suitable materials will be readily understood to those of skill in the art. A preferred material is porous polyethylene, such as types that are commercially available from General Polymetrics (Reading, PA).

A substrate can be readily bound to the structure, such as attachment factors, angiogenic factors, or EGA. Such materials will promote the aggregation of cell culture to the structure. Appropriate attachment factors include, for example, collagen, fibronectin, laminin, to name a few. Also, angiogenic factors preferred for use in the invention include, angiogenin or acidic FGF.

The structure, complete with the substrate, is immersed in a cell culture containing liver cells and supplemented with EGA as described above. The cells will attach and grow on the structure.

The reconstituted liver cells can be used in liver grafting techniques or for the production of biological products normally formed in the liver.

Example 26.

In Vivo Liver Cell Reconstitution with EGA

The solid phase implantable supporting structure prepared

in accordance with Example 25 can be implanted in a patient's liver in order to stimulate regrowth of liver cells. In a preferred embodiment, the supporting structure is infused with an external source of EGA in order to provide continued growth enhancement opportunities for the cells on the support as well as neighboring tissue. Infusion is accomplished in a preferred embodiment through an osmotic pump, for example.

In another preferred embodiment, the production of EGA is made systemic to the supporting structure, through, for example, attaching cells that are active producers and excreters of EGA.

While the present invention has been described above with reference to the preferred embodiments thereof, it should be apparent to those skilled in the art that various modifications and changes in the process can be incorporated without departing from the true spirit of the invention as defined in the attached claims.

WHAT IS CLAIMED IS:

1. A growth factor comprising enzymatically glycosylated albumin of the type occurring in the hepatoma cell line HEP G2 wherein said growth factor is substantially purified and is active in promoting the growth of mammalian cells *in vitro*.

2. An enzymatically glycosylated albumin in isolated or purified form comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity.

3. The glycosylated albumin according to Claim 2, derived from bodily fluids.

4. The glycosylated albumin according to Claim 3, wherein the bodily fluids are mammalian.

5. The glycosylated albumin according to Claim 4, wherein the mammalian fluids are human.

6. The glycosylated albumin according to Claim 2, derived from cell, tissue or organ culture, *in vitro*.

7. The glycosylated albumin according to Claim 6, wherein the culture comprises hepatoma cells.

8. The glycosylated albumin according to Claim 2, wherein the growth promoting and/or modulating activity comprises said albumin binding to cells.

9. The glycosylated albumin according to Claim 8, wherein binding is related to a particular growth phase.

10. The glycosylated albumin according to Claim 2, wherein the oligosaccharide moiety comprises sialic acid or N-acetylglucosamine moieties.

11. The glycosylated albumin according to Claim 2, comprising lectin-binding activity.

12. The glycosylated albumin according to Claim 11, wherein the lectin binding activity comprises wheat germ lectin (T. vulgaris) or elderbark lectin Sambucus nigra.

13. The glycosylated albumin according to Claim 5, wherein the human bodily fluid-derived glycosylated albumin comprises an N-terminal amino acid sequence identical with

human serum albumin.

14. The glycosylated albumin according to Claim 2, comprising serological similarity with albumin.

15. The growth factor of Claim 1, wherein the glycosylated albumin comprises:

(a) an apparent M.W. of at least 65,000 daltons on SDS/PAGE;

(b) serologically identified albumin;

(c) binding to N-acetyl-D-glucosamine or sialic acid specific lectins;

(d) deglycosylated and/or desialylated by specific glycosidases;

(e) binding to a cell surface receptor; and

(f) activating and/or modulating cell growth.

16. A composition for cell, tissue or organ culture media comprising the growth factor according to Claim 15 in combination with an aqueous growth-supporting medium.

17. The composition for cell, tissue or organ culture media according to Claim 16 which are serum-free.

18. An *in vitro* diagnostic test of liver function and/or pathology thereof comprising the steps of:

obtaining a sample of serum or plasma from an animal;

isolating the protein fraction therefrom that has the properties of the glycosylated albumin according to Claim 15; and

quantitating the amount of said glycosylated albumin in comparison to a known quantity.

19. A diagnostic test of liver function and/or pathology thereof comprising the use of the glycosylated albumin according to Claim 15.

20. A glycoprotein isolated from mammalian sources comprising an apparent molecular weight of at least 65,000 daltons on SDS/PAGE, identified serologically and enzymatically as a glycosylated form of albumin having growth promoting activity.

21. The glycoprotein according to Claim 19 comprising a product of a hepatoma cell culture.

22. The glycoprotein according to Claim 20 wherein the product is isolated from conditioned media of the hepatoma cell culture.

23. A pharmaceutical composition comprising the glycosylated albumin of Claim 1 and a pharmaceutical acceptable carrier.

24. A method of topical or percutaneous treatment of tissue that is in need of growth factor treatment in a warmblooded animal, which comprises administering to the animal an effective amount of the glycosylated albumin according to Claim 1 with a pharmaceutically acceptable carrier in a manner designed to deliver said glycosylated albumin to the tissue in need of treatment.

25. A process for purifying the glycosylated albumin of Claim 1 which comprises subjecting a sample to be purified to one or more of salt precipitation; ion exchange chromatography; gel filtration; molecular sieve filtration or dialysis; lectin affinity chromatography; and gel electrophoresis.

26. A method for determining whether a fraction of a fluid contains an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity, comprising:

inoculating a mammalian cell culture with said fraction;

incubating said cell culture containing said fraction; and

determining the presence or absence of said enzymatically glycosylated albumin based on the growth of said cells,

wherein an increase in the growth of the cells indicates the presence of the enzymatically glycosylated albumin and a lack of growth indicates the absence of the enzymatically

glycosylated albumin.

27. An isolated neutralizing or inhibiting antibody against the growth factor of Claim 1.

28. An isolated neutralizing or inhibiting antibody against an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

29. Use of the neutralizing or inhibiting antibody of Claim 28 in the immunoaffinity purification of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

30. Use of the neutralizing or inhibiting antibody of Claim 28 in the treatment of a disease state wherein an enzymatically glycosylated albumin is overproduced, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

31. An isolated antibody against the growth factor of Claim 1.

32. An isolated antibody against an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

33. Use of the antibody of Claim 32 in the immunoaffinity purification of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin omprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

34. Use of the antibody of Claim 32 to specifically label cells involved in the production of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an

albumin and further comprising mammalian cell growth promoting and/or modulating activity.

35. A method to specifically target cells in a patient involved in the production of an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least on oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity, comprising:

chemically linking the antibody of Claim 32 to radionuclide to form a radiolabelled antibody; and

administering said radiolabelled antibody to the patient,

wherein the administration results in the delivery of the radiolabelled antibody to said target cells.

36. A method to purify an enzymatically glycosylated albumin from liquid fraction, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further comprising mammalian cell growth promoting and/or modulating activity, comprising the steps of:

providing a column containing a packing material linked to the antibody of Claim 32;

passing the liquid fraction through the column under conditions wherein said enzymatically glycosylated albumin preferentially binds to the antibodies bound to said column; and

eluting said enzymatically glycosylated albumin from the column.

37. A hybridoma cell line that secretes an inhibiting or a neutralizing antibody to an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having mammalian cell growth promoting and/or modulating activity.

38. A hybridoma cell line that secretes an antibody against an enzymatically glycosylated albumin, said enzymatically glycosylated albumin comprising at least one oligosaccharide moiety linked to an albumin and further having

mammalian cell growth promoting and/or modulating activity.

TWO PARAMETER ANALYSIS

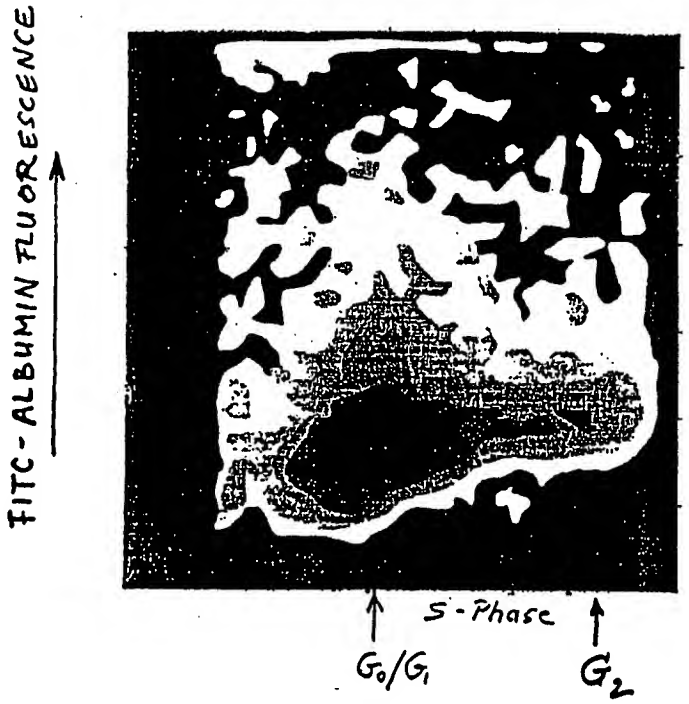


FIG. 1

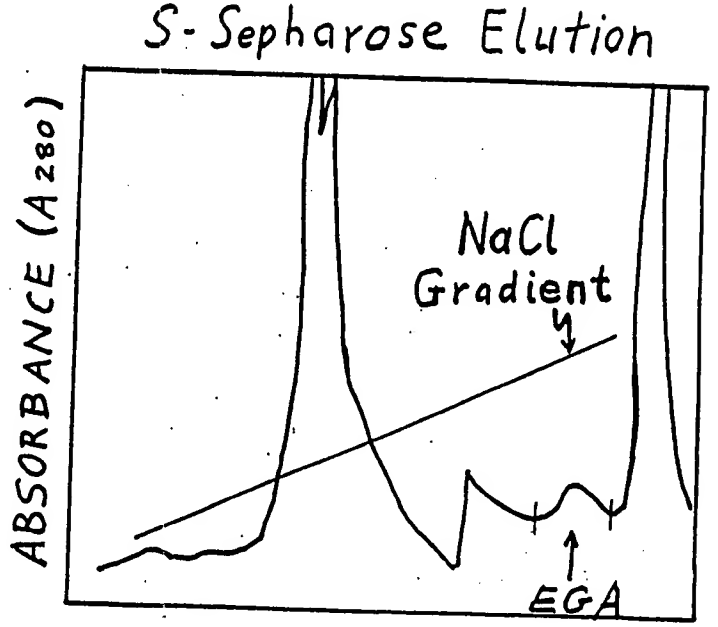
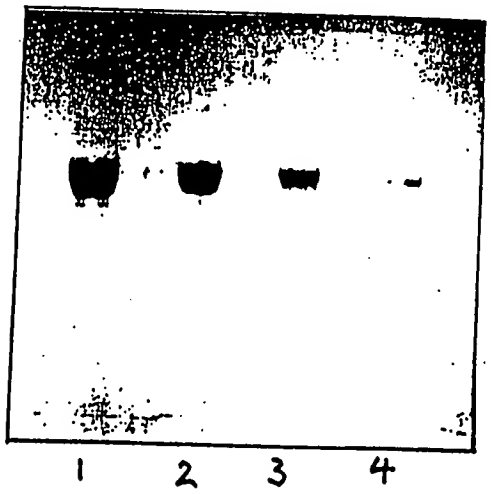


FIG. 2



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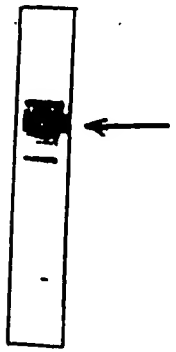


FIG. 4

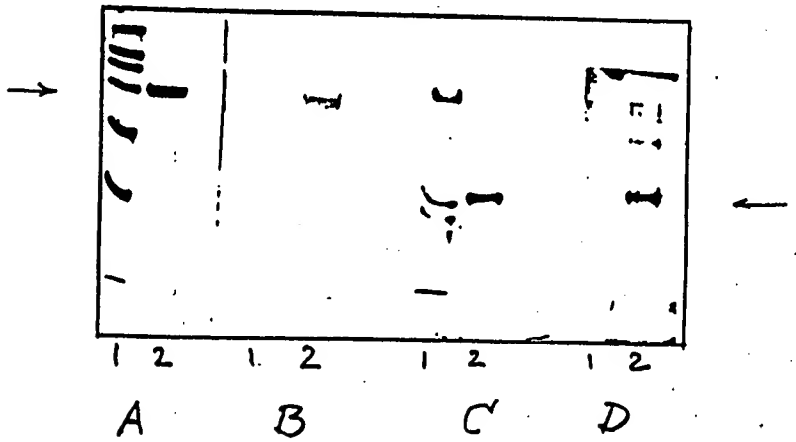
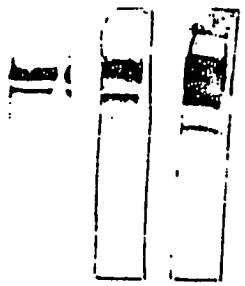


FIG. 5



1 2 3

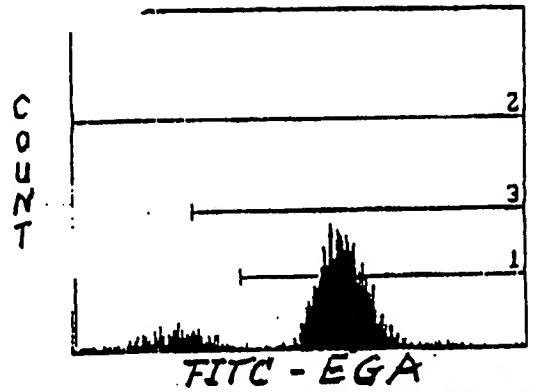
FIG. 6

A

B

FIG. 7

6 / 7



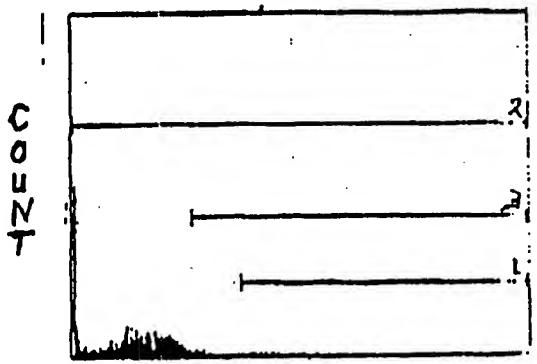


FIG. 8

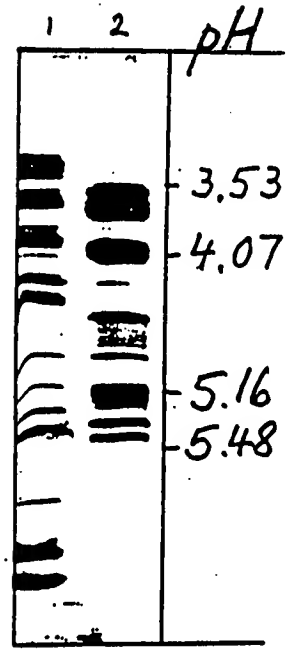


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/01739

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 15/06, A61K 37/36, A61K 37/02 // A 61 K 35/16
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP, A2, 0241830 (THE GENERAL HOSPITAL CORPORATION), 21 October 1987 (21.10.87) --	1-23,25,36
A	Dialog Information Services, file 154, Dialog Acc.no. 07797157, Peach R.J. et al: "Structural characterization of a glycoprotein variant of human serum albumin: albumin Casebrook (494 Asp---Asn.)", & Biochim Biophys Acta Jul 26 1991, 1097 (1) p49-54 --	1-23,25,36

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1993	27. 07. 93
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer MIKAEL G:SON BERGSTRAND

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PCT/US 93/01739

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Proc. Natl. Acad. Sci. USA, Volume 87, February 1990, Andrzej Mackiewicz et al, "Transforming growth factor betal regulates production of acutephase proteins" -- ---------	1-23,25,36

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/01739

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 24,30,35
because they relate to subject matter not required to be searched by this Authority, namely:

Methods for treatment of the human or animal body, c.f. PCT rule 39 (iv).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant; this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/05/93

International application No.
PCT/US 93/01739

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0241830	21/10/87	AU-A- 7301787	09/11/87
		JP-T- 1500188	26/01/89
		WO-A- 8706239	22/10/87